Transcript Profiling in the *chl1-5* Mutant of Arabidopsis Reveals a Role of the Nitrate Transporter NRT1.1 in the Regulation of Another Nitrate Transporter, NRT2.1 [W]

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Arabidopsis thaliana mutants deficient for the NRT1.1 NO_3^- transporter display complex phenotypes, including lowered NO_3^- uptake, altered development of nascent organs, and reduced stomatal opening. To obtain further insight at the molecular level on the multiple physiological functions of NRT1.1, we performed large-scale transcript profiling by serial analysis of gene expression in the roots of the *chl1-5* deletion mutant of *NRT1.1* and of the Columbia wild type. Several hundred genes were differentially expressed between the two genotypes, when plants were grown on NH_4NO_3 as N source. Among these genes, the N satiety-repressed *NRT2.1* gene, encoding a major component of the root high-affinity NO_3^- transport system (HATS), was found to be strongly derepressed in the *chl1-5* mutant (as well as in other *NRT1.1* mutants). This was associated with a marked stimulation of the NO_3^- HATS activity in the mutant, suggesting adaptive response to a possible N limitation resulting from *NRT1.1* mutation. However, derepression of *NRT2.1* in NH_4NO_3-fed *chl1-5* plants could not be attributed to lowered production of N metabolites. Rather, the results show that normal regulation of *NRT2.1* expression is strongly altered in the *chl1-5* mutant, where this gene is no more repressible by high N provision to the plant. This indicates that NRT1.1 plays an unexpected but important role in the regulation of both *NRT2.1* expression and NO_3^- HATS activity. Overexpression of *NRT2.1* was also found in wild-type plants supplied with 1 mM NH_4^+ plus 0.1 mM NO_3^-, a situation where NRT1.1 is likely to mediate very low NO_3^- transport. Thus, we suggest that it is the lack of NRT1.1 activity, rather than the absence of this transporter, that derepresses *NRT2.1* expression in the presence of NH_4^+. Two hypotheses are discussed to explain these results: (1) *NRT2.1* is upregulated by a NO_3^- demand signaling, indirectly triggered by lack of NRT1.1-mediated uptake, which overrides feedback repression by N metabolites, and (2) NRT1.1 plays a more direct signaling role, and its transport activity generates an unknown signal required for *NRT2.1* repression by N metabolites. Both mechanisms would warrant that either NRT1.1 or NRT2.1 ensure significant NO_3^- uptake in the presence of NH_4^+ in the external medium, which is crucial to prevent the detrimental effects of pure NH_4^+ nutrition.

INTRODUCTION

The acquisition of nitrogen by plant roots mostly relies on the activity of NO_3^- and NH_4^+ transport systems located at the plasma membrane of root cells. For both NO_3^- and NH_4^+, these transport systems are functionally separated in high-affinity transport systems (HATS), mediating N uptake in the low external concentration range (<0.5 mM), and low-affinity transport systems (LATS), predominantly active in the high external concen-

tration range (>0.5 mM). To date, the genes encoding NO_3^- or NH_4^+ transporters have been found in four different families; namely, *NRT1* and *NRT2* families for NO_3^- transporters (Forde, 2000) and *AMT1* and *AMT2* families for NH_4^+ transporters (von Wirén et al., 2000). Concerning more specifically NO_3^- transport in *Arabidopsis thaliana*, the *NRT2* family includes seven genes (Orsel et al., 2002), but the *NRT1* family is more difficult to define precisely. It has been restricted to four genes in previous studies (Okamoto et al., 2003), but these genes belong to the large PTR family of transporters, with 51 members. To date, nothing excludes the possibility that NO_3^- transporters are also encoded by other *PTR* genes than the four *NRT1* initially investigated. Of these 58 putative transporters (seven NRT2 and 51 PTR), only three (NRT1.1, NRT1.2, and NRT2.1) have been functionally characterized in planta and shown to ensure part of the NO_3^- uptake from the external medium. NRT1.1 (also called CHL1) was the first NO_3^- transporter identified in plants (Tsay et al., 1993) and was initially believed to be a NO_3^- inducible low-affinity transporter (Tsay et al., 1993; Huang et al., 1996; Touraine and Glass, 1997). In the same family as NRT1.1, NRT1.2 was also characterized as a low-affinity NO_3^- transporter, but with a

constitutive expression, not dependent on the presence of NO_3^- (Huang et al., 1999). Thus, both NRT1.1 and NRT1.2 were considered to belong to the NO_3^- LATS in *A. thaliana* (Crawford and Glass, 1998). These two transporters do not fulfill similar functions because *NRT1.1* mutants appear to be strongly defective in LATS activity only when plants are supplied with a mixed $NO_3^- + NH_4^+$ N source (Touraine and Glass, 1997; Crawford and Glass, 1998), whereas antisense lines of *NRT1.2* also display a markedly reduced LATS activity on NO_3^- as sole N source (Huang et al., 1999). On the other hand, the *NRT2.1* gene was shown to encode a major component of the NO_3^- HATS (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Filleur et al., 2001). It appears to play a crucial role in the control of the high-affinity NO_3^- uptake by the plant because its expression is regulated as the NO_3^- HATS is. For example, it is inducible by NO_3^- itself (Filleur and Daniel Vedele, 1999; Zhuo et al., 1999; Nazoa et al., 2003), repressed by reduced N metabolites (Lejay et al., 1999; Zhuo et al., 1999), and stimulated by photosynthates (Lejay et al., 1999, 2003). Although NRT1.1, NRT1.2, and NRT2.1 do not mediate all NO_3^- transport steps occurring in the plant, these three transporters have a central importance for NO_3^- acquisition in *A. thaliana*. Hence, in addition to the study of some of the other 55 putative transporters (Orsel et al., 2002; Okamoto et al., 2003), further analysis of the overall function of NRT1.1, NRT1.2, and NRT2.1 is still needed. Concerning NRT1.1, the picture becomes more and more complex with recent reports indicating that the role of this transporter is far from being limited to low-affinity NO_3^- uptake in roots. First, NRT1.1 is now considered as a dual affinity transporter, belonging to both HATS and LATS (Wang et al., 1998; Liu et al., 1999). Phosphorylation of NRT1.1, triggered by limited external NO_3^- availability, is responsible for the shift from low to high affinity, thus adapting the functional properties of the transporter to the resource level in the root environment (Liu and Tsay, 2003). Second, NRT1.1 is strongly expressed in nascent organs of both root and shoot (root tip, emerging lateral roots, and nascent leaves) and plays a crucial role in the early phases of development of these young organs (Guo et al., 2001). In particular, several NRT1.1 mutants display altered root architecture in some conditions, with a reduced growth of both primary and secondary roots, sometimes even in the absence of added NO_3^- in the external medium. This suggests an alternative function for NRT1.1, independent of NO_3^- transport (Guo et al., 2001). Finally, it has been reported recently that the mutation of *NRT1.1* also leads to a lower sensitivity to drought, related to reduced stomatal opening because of impaired NO_3^- transport in stomata guard cells (Guo et al., 2003). Clearly, the view that NRT1.1 behaves only as a transporter in charge of the NO_3^- uptake from the external medium is an oversimplification. This protein appears to fulfill a multiplicity of physiological functions, which begin to be unravelled more than 30 years after the identification of the first *NRT1.1* mutant (Oostindiër-Braaksma and Feenstra, 1973).

Most of the novel and important findings mentioned above concerning *NRT1.1* rely on physiological or morphological analyses of mutants. Very few molecular data are associated with these reports, thus resulting in a lack of understanding of the gene networks functionally associated with *NRT1.1* in the control of N acquisition, root and shoot development, and water use in the plant. To obtain further insight on this point, we performed large-scale transcript profiling in roots of both the *chl1-5* mutant of *NRT1.1* (Tsay et al., 1993) and the related Columbia (Col) wild type. Our transcriptomic approach was based on the serial analysis of gene expression (SAGE) methodology (Velculescu et al., 1995), which involves the generation of a short specific tag (14 bp) for each mRNA in a sample. The sequencing of a large number of SAGE tags in a sample library allows a high-throughput analysis of the frequencies of these tags, which are representative of the relative amounts of the corresponding mRNAs. Thus, the comparison of the tag sequences and copy numbers obtained from two different libraries allows the identification of the genes differentially expressed between the two original samples. SAGE has been mostly employed in cancer research (Boon et al., 2002) but is now increasingly used in plants (Lorenz and Dean, 2002; Matsumura et al., 2003), especially in *A. thaliana* (Jung et al., 2003; Lee and Lee, 2003), in which the full genome sequence provides a unique tool for identifying the genes corresponding to the tags found experimentally (Fizames et al., 2004).

In addition to the finding that many genes show a markedly altered level of expression in the roots of the *chl1-5* mutant as compared with the Col wild type, we report here the observation that *NRT2.1* expression is markedly deregulated in the mutant, a response that could not be explained by the known regulation affecting this gene. This suggests either the occurrence of a yet unknown signaling for control of *NRT2.1* expression or a role of NRT1.1 in the regulation of other NO_3^- transporters at the gene expression level.

RESULTS

### Comparison of SAGE Libraries from Col and *chl1-5* Roots

Because NRT1.1 has been shown to be the major NO_3^- transporter involved in root NO_3^- uptake under mixed N nutrition (NO_3^- plus NH_4^+; Touraine and Glass, 1997; Crawford and Glass, 1998), the two SAGE libraries were generated from roots of Col-0 and *chl1-5* plants grown hydroponically on 1 mM NH_4NO_3. These libraries were sequenced up to 31,354 and 28,451 tags for Col-0 and *chl1-5* roots, respectively (the Col-0 library has already been reported under the name of the NH_4NO_3 library in Fizames et al., 2004). The 59,805 total tags correspond to 25,230 different sequences, among which 7583 are represented by tags found at least twice and up to 228 times. Because of rare but unavoidable sequencing and PCR errors, the use of single tags is not totally safe for gene identification. Thus, we restricted our analysis to the 7583 different tags found at least twice in the two combined libraries. The identification of the genes represented in our SAGE transcriptomes was performed by matching the list of the 7583 experimental tags to that of the virtual ones obtained by extracting the predicted SAGE tag sequence from each gene annotated in the whole Arabidopsis genome (Fizames et al., 2004). Among the 7583 different experimental tags, 1972 had no gene match in the database of virtual SAGE tags, 885 matched several genes and were thus not specific, and 4726 matched one single gene. The whole set of data on the 5611 tags matching one or several genes can be

viewed online (http://genoplante-info.infobiogen.fr; see Supplemental Table 1 online).

The statistical analysis of the comparison between Col and *chl1-5* libraries (Figure 1) resulted in the identification of 419 tags with different copy numbers in the two libraries at P < 0.01 (1194 tags at P < 0.05). Among these, 296 tags could be attributed to one single gene at P < 0.01 (797 tags at P < 0.05). The 296 differentially expressed genes (P < 0.01) reveal a large variety of functions affected in the *chl1-5* mutant (http://genoplante-info.infobiogen.fr; see Supplemental Table 2 online) but also include genes directly related either to N nutrition or ion transport (Table 1). A few genes encoding enzymes of N metabolism have a strongly altered expression. This is the case of two isoforms of glutamate dehydrogenase (GDH1 and GDH2), which are markedly underexpressed in roots of *chl1-5* compared with Col. Several transporter or channel genes also show changes in expression between the two genotypes. Of particular interest are those related to NO_3^- or amino acid transport, such as *NRT2.1*, At1g32450 (a member of the large *PTR* multigene family including *NRT1.1*), At4g38250, and *AAP6*, which are all strongly overexpressed in *chl1-5*. On the other hand, genes encoding aquaporins (*PIP2;2*, *PIP1;2*, *PIP2;1*, and *PIP1;1*) and metal (*IRT1* and *NRAMP1*), SO_4^{2-} (*SULTR1;2*), or K^+ (*SKOR*) transporters/channels are repressed in the mutant.

To investigate the reliability of the SAGE data, RNA gel blot analysis was performed on eight selected genes, with the same samples as those used for the construction of the SAGE libraries. The genes investigated corresponded to N assimilation-related

genes or ion channel or transporter genes, either found to be differentially expressed (Table 1) or not. These genes include the following: *NRT2.1* (encoding a high-affinity NO_3^- transporter; Filleur et al., 2001), *NIA1* and *NIA2* (encoding the two nitrate reductase [NR] isoforms present in *A. thaliana*; Wilkinson and Crawford, 1993), *GS2* (encoding the chloroplastic isoform of Gln synthetase; Peterman and Goodman, 1991), *AMT1.1* (encoding a high-affinity NH_4^+ transporter; Ninnemann et al., 1994), *SKOR* (encoding a K^+ channel implicated in xylem loading; Gaymard et al., 1998), and *IRT1* (encoding an iron transporter; Vert et al., 2002). The absence of the *NRT1.1* SAGE tag in the *chl1-5* library could not be verified because this tag is not specific and also matches nine other genes, but as expected, *NRT1.1* transcript was not detected in the *chl1-5* mutant (data not shown). *NIA1*, *NIA2*, *GS2*, and *AMT1.1* were not found in the list of differentially expressed genes and did not show any significant difference in their transcript accumulation between Col and *chl1-5* roots (Figure 2). The slightly higher mRNA levels apparent in Figure 2 for *NIA1* and *NIA2* in the mutant are not representative. In four independent experiments, the *chl1-5*/Col ratio was measured at 0.81 ± 0.25 and 1.37 ± 0.38 for transcript accumulation of *NIA1* and *NIA2*, respectively. At the opposite, *NRT2.1* was found by SAGE to be significantly overexpressed in *chl1-5* roots as compared with Col roots (Table 1). This marked difference in transcript accumulation was confirmed by RNA gel blot analysis (Figure 2), with approximately the same ratio of six between Col and *chl1-5* (mean value for this ratio in five independent experiments: 6.51 ± 1.76). For both *SKOR* and *IRT1*, the SAGE data
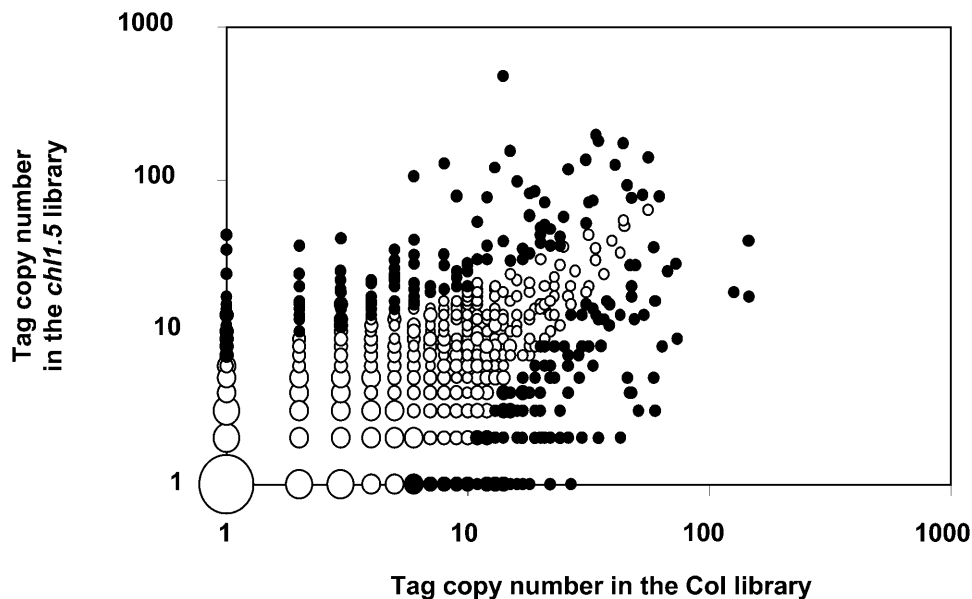
Figure 1. Scatter Plot of Tag Frequencies in Col and *chl1-5* SAGE Libraries.

The two libraries were obtained from roots of 6-week-old plants grown hydroponically on complete nutrient solution containing 1 mM NH_4NO_3 as N source. A total of 31,354 and 28,451 tags were sequenced for Col-0 and *chl1-5*, respectively. The tags with no occurrence in one library were set at a copy number of one in this library to enable their representation on a logarithmic scale. The size of the data points is correlated with the number of different tags with the same coordinates. The closed symbols correspond to tags with frequencies significantly different (P < 0.01) between the two libraries.

Table 1. Expression of Genes Related to Nitrogen Metabolism and Ion Transport in Col and *chl1-5* Roots

Gene	SAGE Tag (5′/3′)	Tag Copy Number in Col	Tag Copy Number in *chl1-5*	Probability	Function
At5g07440	GATCCAGATGCTGA	26	4	1.27E-05	Glu dehydrogenase (GDH2)
At5g54810	GATCGGTGTGGAAG	29	7	6.07E-05	Trp synthase TSB1 (β subunit)
At5g17330	GATCGATATAGAGA	10	0	4.88E-04	Glu decarboxylase (GAD1)
At5g18170	GATCTCCGGATGGG	9	0	9.76E-04	Glu dehydrogenase (GDH1)
At5g19550	GATCTACCGTTTCT	8	19	8.27E-03	Asp aminotransferase (ASP2)
At5g11520	GATCGTATCATCAG	7	1	0.015	Asp aminotransferase (ASP3)
At3g47340	GATCGATGTACATC	0	4	0.031	Asn synthetase (ASN1)
At2g37170	GATCCTTCAGAAGT	29	5	8.09E-06	Aquaporin (PIP2;2)
At2g45960	GATCTTCGCTCTCG	43	13	1.31E-05	Aquaporin (PIP1;2)
At1g32450	GATCAGTCTTTTTC	2	23	1.83E-04	Nitrate/peptide transporter, putative
At1g08090	GATCGCATATAAGA	3	18	3.17E-04	Nitrate transporter (NRT2;1)
At3g02850	GATCCAATTGGTAG	7	0	3.91E-03	Stelar K^+ channel (SKOR)
At4g17340	GATCAATCCTATAG	0	10	4.88E-04	Aquaporin (TIP2;2)
At4g19690	GATCTATCACATTT	9	1	4.88E-03	Fe transporter (IRT1)
At1g80830	GATCTTCGTAGGAA	6	0	7.81E-03	Metal ion transporter (NRAMP1)
At3g53420	GATCTCTCTGTACA	27	15	0.011	Aquaporin (PIP2;1)
At5g13750	GATCCAAAGTTAGA	5	0	0.015	Transporter-like protein
At5g14040	GATCGGGACGTTGA	1	7	0.016	Mitochondrial phosphate translocator
At1g78000	GATCCAGAGATGGC	10	3	0.017	Sulphate transporter (Sultr1;2)
At3g61430	GATCTACTACATGT	26	17	0.024	Aquaporin (PIP1;1)
At4g38250	GATCAGCTCTGTCT	1	6	0.027	Putative amino acid transporter
At5g49630	GATCAGTGCAAGGA	1	5	0.047	Amino acid transporter (AAP6)

suggested a lower expression level in roots of *chl1-5* than in those of Col, which was also verified by RNA gel blot analysis (Figure 2).

Higher Expression of *NRT2.1* Is Associated with Upregulation of the High-Affinity NO_3^- Uptake System in the *chl1-5* Mutant

The above data reveal complex molecular responses to the mutation affecting *chl1-5*. However, among all the gene responses identified, the finding that *NRT2.1* was markedly overexpressed in the *chl1-5* mutant as compared with Col was highly surprising. Indeed, *NRT2.1* is a major component of the NO_3^- HATS and is strongly repressed when NH_4^+ is present in the nutrient solution (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001) (i.e., under the conditions of our study). Thus, the totally unexpected observation of a high *NRT2.1* expression level in *chl1-5* in the presence of NH_4^+ prompted us to focus further investigation on this intriguing point.

Numerous reports have shown a strong correlation between *NRT2.1* transcript accumulation in the roots and the activity of the HATS for NO_3^- (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Gansel et al., 2001; Okamoto et al., 2003). Thus, we investigated if *NRT2.1* overexpression in the *chl1-5* mutant also had functional consequences on NO_3^- uptake rate by this mutant. To do so, the kinetics of $^{15}NO_3^-$ influx as a function of external $^{15}NO_3^-$ concentration was determined in both Col and *chl1-5* plants grown for 6 weeks on 1 mM NH_4NO_3 (Figure 3A). In the low NO_3^- concentration range (10 to 500 μM), $^{15}NO_3^-$ influx in *chl1-5* was higher than in Col, whereas in the high concentration range (0.5 to 5 mM), the reverse was observed with $^{15}NO_3^-$

influx in *chl1-5* roots lower than in Col. The stimulation of the HATS activity in *chl1-5* as compared with Col was most pronounced at 25 to 50 μM external $^{15}NO_3^-$ (approximately fourfold increase; Figure 3B). This is the exact range of concentration where NRT2.1 was shown to participate predominantly to root NO_3^- uptake (Cerezo et al., 2001), indicating that the upregulation of the HATS in *chl1-5* plants was most probably attributable to the overexpression of *NRT2.1* as compared with Col.

Characterization of the *chl1-5* Deletion and Isolation of T-DNA Insertion Mutants for *NRT1.1*

The size and location of the deletion affecting the *chl1-5* mutant has not been reported, despite the extensive use of this genotype for functional characterization of the NRT1.1 transporter (Huang et al., 1996; Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999; Guo et al., 2001). To determine whether other genes than *NRT1.1* are also absent in *chl1-5*, we used a PCR approach to map the deletion. Successive PCR amplifications were performed on Col and *chl1-5* genomic DNA using oligonucleotides designed from the sequence of chromosome 1 (GenBank accession number NC_003070). This revealed that the *chl1-5* deletion corresponds to an 18.31-kb DNA fragment, beginning in the last *NRT1.1* intron and ending after the At1g12090 gene (Figure 4). These data show that not only *NRT1.1* but also two other genes, At1g12090 and At1g12100, are affected in the *chl1-5* mutant. These two genes are highly homologous. No EST was found for the At1g12100 gene in all available databases, and its SAGE tag was absent from both Col and *chl1-5* libraries. The At1g12090 gene seems to be significantly expressed because many ESTs were found, with some of
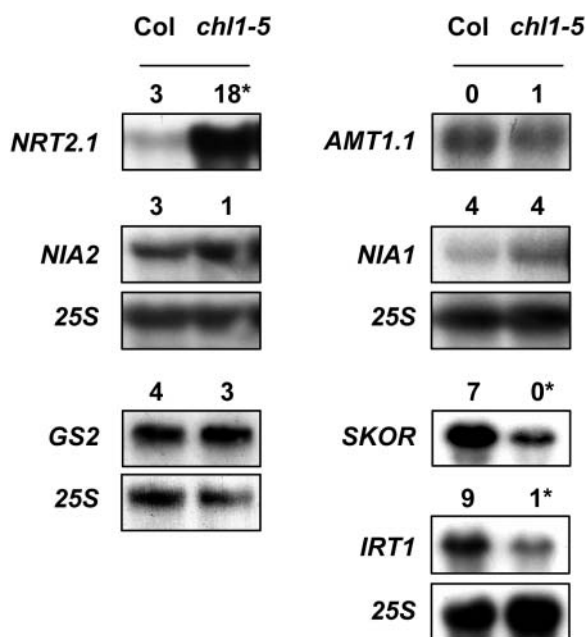
Figure 2. Gel Blot Analysis of Transcript Accumulation of Various N Metabolism or Transporter Genes between Roots of Col or *chl1-5* Plants.

The tag copy numbers of the respective SAGE tags of these genes are indicated on top of the autoradiograms for comparison. The asterisks indicate difference between tag copy number in Col and *chl1-5* statistically significant at P < 0.01. The experimental conditions are those described in Figure 1.

them corresponding to a root specific library. The SAGE tag of At1g12090 was recorded four times in the Col library, but as expected, was not found in the *chl1-5* library.

As a consequence, it cannot be ruled out that part of the *chl1-5* phenotype is a result of the deletion of either the At1g12090 or the At1g12100 gene. To safely attribute specific aspects of this phenotype to the deletion of *NRT1.1*, we then searched for other *NRT1.1*-null mutants. Two additional mutants (*chl1-10* and *chl1-11*) were isolated from the T-DNA tagged lines collection of INRA Versailles by a chlorate resistance screen. Both mutants belong to the *chl1-5* complementation group (data not shown) and carry a T-DNA insertion in *NRT1.1* (determined by DNA gel blot analysis). The *chl1-10* mutant has a unique T-DNA insertion, which was located in the beginning of the last exon of *NRT1.1* (between nucleotides 3130 and 3132 after the initiation codon). The *chl1-11* mutant also has three other T-DNA insertions.

Regulation of *NRT2.1* Expression by N Status of the Plant Is Altered in *NRT1.1* Mutants

The overexpression of *NRT2.1* observed in NH_4NO_3-grown *chl1-5* plants was also found in three other *NRT1.1* mutants (Figure 5): *chl1-10, chl1-11*, and the original *chl1-1* mutant (formerly called B1; Doddema and Telkamp, 1979). This demonstrates that upregulation of *NRT2.1* expression is specifically attributable to the *NRT1.1* mutation. Moreover,

the comparison of wild-type and mutant plants either grown on NO_3^- or NH_4NO_3 showed that *NRT2.1* expression was strongly repressed by NH_4^+ in wild-type plants, but surprisingly not in *chl1-1, chl1-10*, and *chl1-11* plants (Figure 5).

Such a lack of downregulation of *NRT2.1* under repressive conditions (NH_4^+ supply) in *NRT1.1* mutants is at odds with the current knowledge of *NRT2.1* regulation. Further work was then
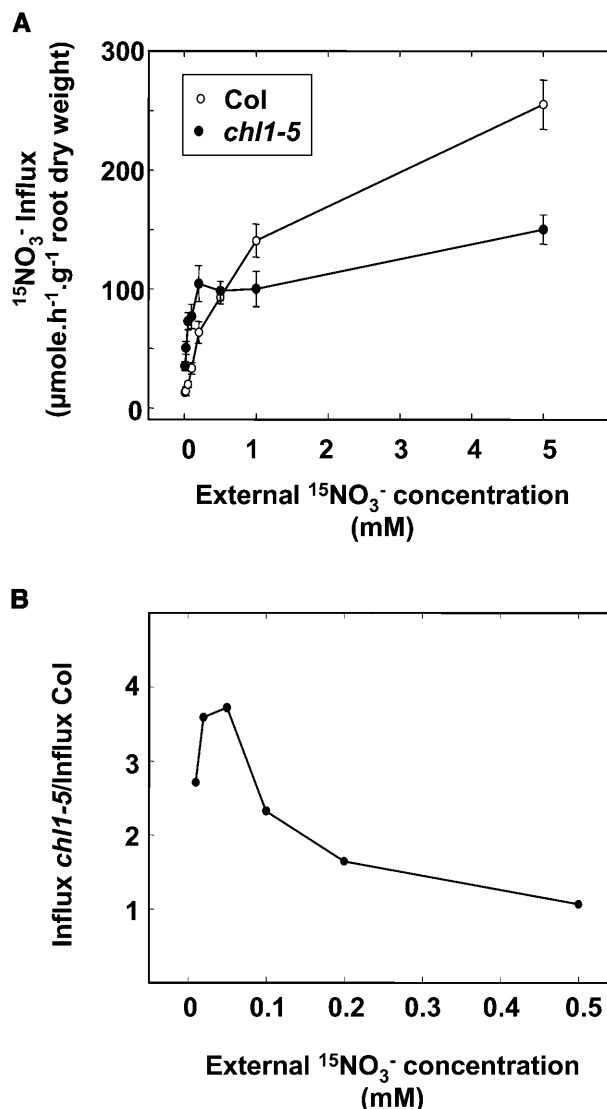

Figure 3. Kinetics of Root $^{15}NO_3^-$ Influx in 6-Week-Old Col and *chl1-5* Plants Grown on Complete Nutrient Solution Containing 1 mM NH_4NO_3 as N Source.

Root $^{15}NO_3^-$ influx was assayed by 5 min labeling in complete nutrient solutions containing $^{15}NO_3^-$ (99 atom percentage ^{15}N) at the concentration indicated.
(A) $^{15}NO_3^-$ influx determined after total ^{15}N analysis in both roots and shoots.
(B) Ratio between $^{15}NO_3^-$ influx in *chl1-5* and Col in the low $^{15}NO_3^-$ concentration range. Values are the mean of 6 to 12 replicates ± SE.
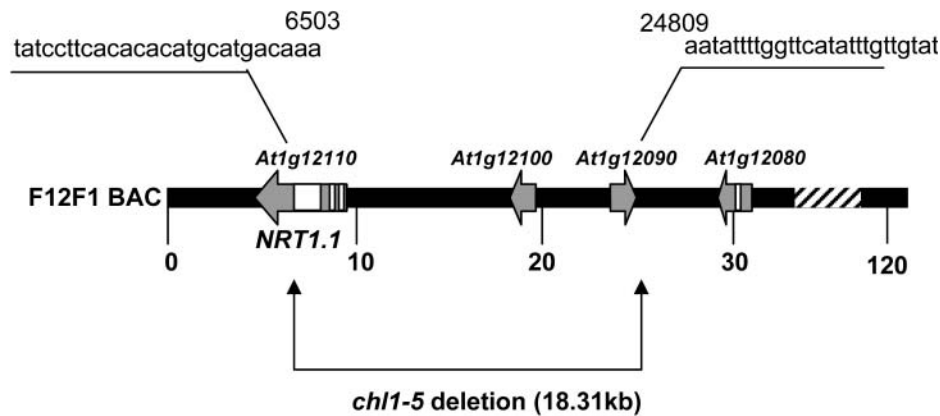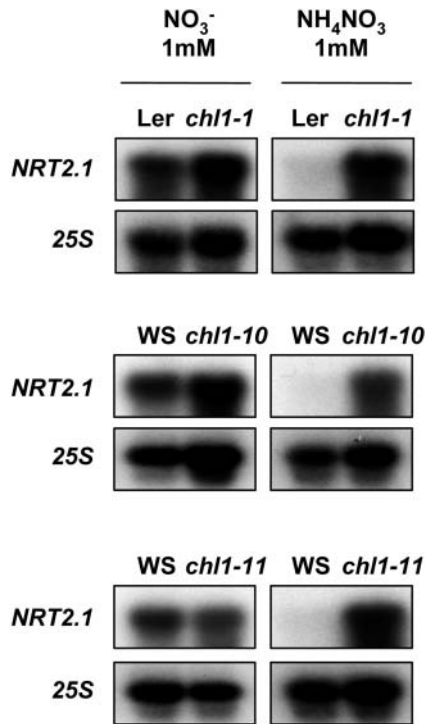
Figure 4. Mapping of the *chl1-5* Deletion on F12F1 BAC (Chromosome 1).

The structure of the corresponding *chl1-5* genomic region was deduced from PCR experiments and sequencing.
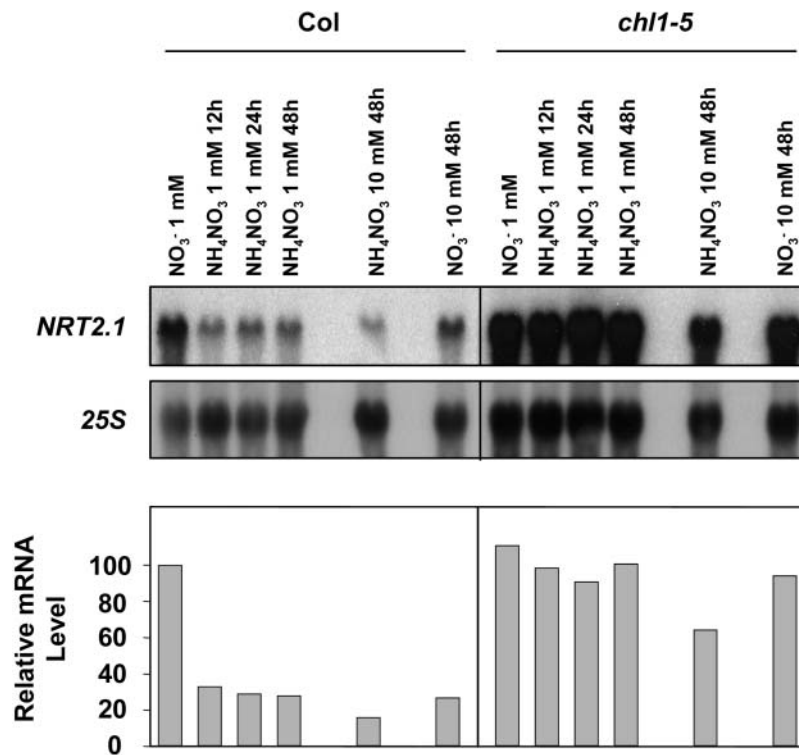
devoted to investigate, in both wild-type and *chl1-5* plants, the main aspects of the control of *NRT2.1* expression, namely, repression by external NH_4^+ or amino acid supply (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001), upregulation in response to N starvation (Filleur and Daniel-Vedele, 1999; Lejay

Figure 5. Gel Blot Analysis of *NRT2.1* Transcript Accumulation in the Roots of *chl1* Mutants.

The plants of the various genotypes were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH_4NO_3 as N source and were either kept on this solution or transferred on another one with 1 mM NO_3^- as N source 1 week before the harvest. Ler, Landsberg *erecta*; WS, Wassilewskija.

et al., 1999), induction by NO_3^- (Filleur and Daniel-Vedele, 1999), and diurnal changes (Lejay et al., 1999).

As previously described (Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001), *NRT2.1* transcript level was rapidly and markedly lowered in wild-type plants by exogenous NH_4^+ supply or high external NO_3^- concentration (Figure 6). This downregulation of *NRT2.1* expression was absent or much less pronounced in *chl1-5* plants (Figure 6). Addition of 5 mM Gln (a strong repressor of *NRT2.1* expression) to the 1 mM NO_3^- medium resulted after 6 h in a nearly 90% decrease of *NRT2.1* transcript level in Col roots, whereas this decrease was only of 50% in *chl1-5* roots (data not shown). Not only the expression of *NRT2.1*, but also the activity of the HATS for NO_3^- was resistant to the repression exerted by a reduced N source in *chl1-5*. Root $^{15}NO_3^-$ influx measured at 0.2 mM external concentration was lowered by ~50% after NH_4^+ supply in Col plants but was unaffected in *chl1-5* plants (Figure 7). Another clear example of altered regulation of *NRT2.1* expression in *chl1-5* plants relates to the response to N starvation (Figure 8). In Col roots, *NRT2.1* transcript level increased 24 and 48 h after transfer of the plants to N-free solution and decreased again thereafter. This transient upregulation has been attributed to the opposite effects of two different regulatory mechanisms (Lejay et al., 1999): relief from repression by N metabolites (initially predominant), on the one hand, and shortage of induction by NO_3^- after several days without NO_3^- supply (predominant after 2 d), on the other hand. In *chl1-5* roots, the initial increase in *NRT2.1* expression after removal of the N source was absent, and only the decay of *NRT2.1* transcript level because of deinduction was observed (Figure 8). Most importantly, this altered response to N starvation is not found for all genes regulated by N status because *AMT1.1*, encoding an N starvation induced NH_4^+ transporter (Gazzarrini et al., 1999; Rawat et al., 1999), displayed a similar upregulation after transfer of the plants to N-free solution in both Col and *chl1-5* roots (Figure 8). The two other main regulations identified for the control of *NRT2.1* expression, namely induction by NO_3^- (Filleur and Daniel-Vedele, 1999) and diurnal changes (Lejay et al., 1999, 2003), are not affected in the *chl1-5* mutant as compared with Col (Figure 9).

Figure 6. Gel Blot Analysis of *NRT2.1* Transcript Accumulation in the Roots of Col and *chl1-5* Plants in Response to Various N Treatments.

The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NO_3^- as N source. At the time of the experiments, one batch of plants was left on 1 mM NO_3^-, and others were transferred for various periods to nutrient solutions with different N sources as indicated in the figure. The relative *NRT2.1* mRNA levels are the means of the values obtained in two replicate experiments and were determined using 25S as a control.

The *chl1-5* Mutant Is Not Affected in NH_4^+ Uptake, Is Not N Deficient When Grown on 1 mM NH_4NO_3, but Accumulates Less NO_3^- Than the Wild Type

We investigated two hypotheses that may explain why *NRT2.1* is upregulated in NH_4NO_3-grown *chl1-5* plants, namely that the *chl1-5* mutant is altered in NH_4^+ uptake or that NH_4NO_3-grown *chl1-5* plants suffer from N deficiency as compared with Col. Assay of $^{15}NH_4^+$ influx at various concentrations in both Col and *chl1-5* plants indicated that *chl1-5* is not deficient for both high- and low-affinity NH_4^+ uptake systems (Figure 10). Indeed, root $^{15}NH_4^+$ influx tended to be slightly higher (5 to 10%) in *chl1-5* than in Col, although the difference was never statistically significant. Total N, NO_3^-, and amino acid contents of roots and shoots were determined to compare the N status of Col and *chl1-5* plants. The hypothesis that *chl1-5* plants grown on 1 mM NH_4NO_3 could be N deficient is contradicted by the fact that total N contents of both roots and shoot were never found to be different between the two genotypes in five independent experiments (data not shown). Furthermore, Gln accumulation in roots of *chl1-5* was almost twice that measured in Col (Figure 11). The accumulation of the other amino acids in roots or of all amino acids in shoots did not differ between the two genotypes. On the other hand, in relation with a lower NO_3^- influx at 1 mM NH_4NO_3 (Figure 3), *chl1-5* plants accumulated less NO_3^- than Col plants

in both roots and shoots (Figure 12). This reduced accumulation of NO_3^- in the mutant was shown to occur also when NO_3^- was the sole N source provided to the plants (Figure 12).

Overexpression of *NRT2.1* in *NRT1.1* Mutant Is Dependent on the External NO_3^-/NH_4^+ Ratio

The above observations may suggest that reduced levels of NO_3^- in tissues of *NRT1.1* mutants can be the cause for over-expression of *NRT2.1*. To investigate this hypothesis, both *chl1-10* and Wassilewskija (Ws) plants were grown for 5 weeks on 1 mM NH_4NO_3, then shifted for 6 d to media containing 1 mM NH_4^+, but with 0.1, 1, or 10 mM NO_3^-. This was expected to alter NO_3^- accumulation in both genotypes, without resulting in N deficiency (because of the presence of 1 mM NH_4^+ in the medium). The modification for 6 d of the external NO_3^- concentration of the medium did not affect NO_3^- levels in the shoots, but resulted in changes in NO_3^- accumulation in the roots (Figure 13). This had no effect on *NRT2.1* expression in *chl1-10* roots, which remained high in all three conditions. Surprisingly, although low at 1 or 10 mM NO_3^-, *NRT2.1* transcript level in Ws roots increased dramatically at 0.1 mM NO_3^- despite the presence of 1 mM NH_4^+ in the medium (Figure 13). In this last situation, *NRT2.1* was not overexpressed any more in *chl1-10*
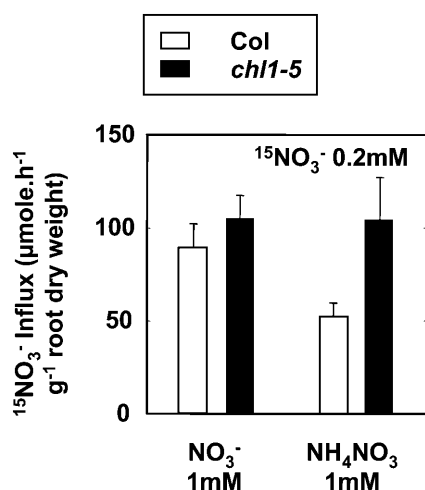
Figure 7. Effect of the Presence of NH_4^+ in the Nutrient Solution on Root $^{15}NO_3^-$ Influx in Col and *chl1-5* Plants.

The plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH_4NO_3 as N source and were either kept on this solution or transferred on another one with 1 mM NO_3^- as N source 1 week before the harvest. Root $^{15}NO_3^-$ influx was assayed by 5 min labeling at 0.2 mM external $^{15}NO_3^-$ concentration. Results are the means of eight replicates ± SE.

compared with the wild type. These data demonstrate that upregulation of *NRT2.1* in *NRT1.1* mutant depends on the external NH_4^+/NO_3^- ratio and that high level of *NRT2.1* expression in the presence of NH_4^+ can occur also in the wild type, in situations of excess NH_4^+ compared with NO_3^-.

DISCUSSION

Our SAGE data indicate that the expression of a high number of genes is modified in the roots of the *chl1-5* mutant as compared with Col. However, despite the extensive use of this mutant to investigate the various aspect of *NRT1.1* function (Huang et al., 1996; Touraine and Glass, 1997; Wang et al., 1998; Liu et al., 1999; Guo et al., 2001, 2003), caution is needed in associating all these changes in gene expression to the mutation of *NRT1.1*. First, transcript profiling was performed in only one series of experiments because of the cost of SAGE. Second, two other genes (At1g12090 and At1g12100) were also found to be deleted in *chl1-5*. Of these two genes, only At1g12090 seems to be expressed in the roots. Its function is unclear because it encodes a protein sharing similarities with pEARLI, an extensin, a protease inhibitor, and AIR1, this latter being possibly involved in the auxin-mediated initiation of lateral roots (Neuteboom et al., 1999). Further work is thus needed, using other *chl1* mutants (Figure 5), to determine the individual genes whose expression is specifically altered by *NRT1.1* mutation. Nevertheless, some of the molecular responses observed in *chl1-5* are correlated with physiological modifications reported in the *chl1-1* mutant, suggesting that they result from *NRT1.1* deletion. For instance,

the fact that various metal/K^+/SO_4^{2-} transporter/channel genes were found to be downregulated in *chl1-5* (Table 1) is consistent with the observation that the *chl1-1* mutant is not only altered in NO_3^- transport but also in the uptake of several other ions (Scholten and Feenstra, 1986).

Concerning NO_3^- uptake, our observation of a lowered LATS activity in the *chl1-5* mutant compared with Col when the plants are supplied with NH_4NO_3 as an N source (Figure 3) is in very good agreement with previous reports on this mutant (Huang et al., 1996; Touraine and Glass, 1997). However, we found this alteration of the LATS compensated for by a much higher HATS activity in *chl1-5* than in Col (Figure 3). These results contrast with previous observations that *chl1-5* and other *chl1* alleles are defective in both HATS and LATS for NO_3^- (Wang et al., 1998; Liu et al., 1999). The reasons for this discrepancy between our results and those of Wang et al. (1998) and Liu et al. (1999) are unclear. However, impaired NO_3^- HATS activity in *NRT1.1* mutants has always been reported in much younger plants (5 to 12 d old) than those used in our study (6 weeks old). Also, many other specific conditions (in particular carbon sources and NO_3^- and NH_4^+ concentrations) were different between our experiments and those of Wang et al. (1998) and Liu et al. (1999) and may explain these contrasting conclusions.

Although a putative NO_3^- transporter gene (At1g32450), uncharacterized to date, is also upregulated in *chl1-5*, we hypothesize that the stimulation of the NO_3^- HATS in the mutant is because of the overexpression of *NRT2.1*. This gene is believed to play a key role in the N acquisition by the roots. It encodes a major component of the HATS for NO_3^- in *A. thaliana*, and its expression is strongly regulated according to N/C status of the plant (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Zhuo et al., 1999; Cerezo et al., 2001; Filleur et al., 2001; Gansel et al., 2001; Lejay et al., 2003). At least three major mechanisms have been proposed to explain the changes in *NRT2.1* transcript accumulation in the root: induction by NO_3^- (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999; Nazoa et al., 2003), induction by
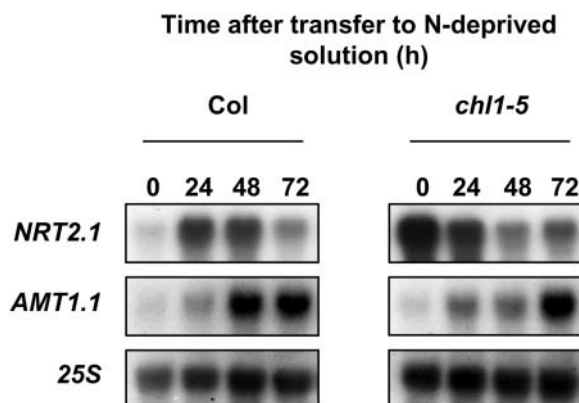
Figure 8. Gel Blot Analysis of *NRT2.1* and *AMT1.1* Transcript Accumulation in the Roots of Col and *chl1-5* Plants in Response to N Starvation.

The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH_4NO_3 as N source before the transfer to an N-deprived medium.

**A**

Time after NO$_3^-$ supply (h)

B

Time of the day
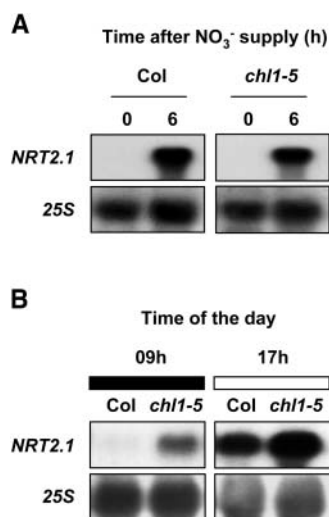
Figure 9. Gel Blot Analysis of *NRT2.1* Transcript Accumulation in the Roots of Col and *chl1-5* Plants in Response to the Induction by NO$_3^-$ and Day/Night Cycle.

The plants were grown hydroponically for 5 **(A)** or 6 **(B)** weeks on complete nutrient solution containing 1 mM NH$_4$NO$_3$ as N source. The plants used for investigating *NRT2.1* induction by NO$_3^-$ **(A)** were transferred for 1 week on an N-deprived medium before the addition of 1 mM NO$_3^-$ in the nutrient solution for 6 h. The plants used for investigating the diurnal changes in *NRT2.1* expression were harvested either at the end of the night (09 h, closed bar) or at the end of the light period (17 h, open bar) **(B)**.

light and sugars (Lejay et al., 1999, 2003), and feedback repression by N metabolites (Lejay et al., 1999; Zhuo et al., 1999; Gansel et al., 2001; Cerezo et al., 2001). Downregulation of *NRT2.1* by N metabolites is postulated to involve products of NO$_3^-$ assimilation, and more particularly NH$_4^+$ and Gln, as negative effectors of the expression of the gene (Lejay et al., 1999; Zhuo et al., 1999; Nazoa et al., 2003). This is expected to ensure the tuning of the HATS activity to the N demand of the whole plant. Accordingly, the deletion of *NRT2.1* (together with part of *NRT2.2*) in the *atnrt2* mutant results in both a lowered activity of the HATS and in the loss of the regulation of this uptake system by the N status of the plant (Cerezo et al., 2001; Filleur et al., 2001).

We show here that the regulation of *NRT2.1* transcript accumulation by N status of the plant is altered in the *chl1-5* mutant (Figures 2, 6, and 8), as well as in other *NRT1.1* mutants (Figures 5 and 13). The first hypothesis that can be considered for explaining the increase in *NRT2.1* transcript level in the *NRT1.1* mutants is that these mutants suffer from N deficiency even when supplied with mixed N sources such as NH$_4$NO$_3$. However, several lines of evidence do not support this hypothesis. First, *chl1-5* plants are not deficient for NH$_4^+$ uptake (Figure 10), and neither the total N influx in roots (NO$_3^-$ plus NH$_4^+$; Figures 3 and 10), nor the total N content of both roots and shoots (data not shown) markedly differ between *chl1-5* and Col plants when grown on 1 mM NH$_4$NO$_3$. Second, the observation that the

accumulation of free Gln in roots is higher in *chl1-5* than in Col (Figure 11) also clearly indicates that *chl1-5* plants are N sufficient. Third, the activity of the NH$_4^+$ HATS in *chl1-5* plants is not derepressed as expected if these plants were N deficient. Root ^{15}NH$_4^+$ influx, measured at 0.2 mM external ^{15}NH$_4^+$, is low ($\sim$50 μmol h^{-1} g^{-1} root dry weight) and similar in both *chl1-5* and Col (Figure 10). In N-limited plants, root ^{15}NH$_4^+$ influx is generally recorded at much higher values (up to 500 μmol h^{-1} g^{-1} root dry weight; Gazzarrini et al., 1999; Rawat et al., 1999). Accordingly, the expression of the N starvation–inducible NH$_4^+$ transporter gene *AMT1.1* (Gazzarrini et al., 1999; Rawat et al., 1999) is low in the *chl1-5* mutant on 1 mM NH$_4$NO$_3$ and not different than in Col (Figure 8). Thus, the overexpression of *NRT2.1* in *chl1-5* cannot be explained by general N deficiency. This suggests that normal regulation of *NRT2.1* expression by N status of the plant is markedly altered in *chl1-5* plants. Indeed, submitting the plants to much more repressive conditions (transfer to 10 mM NO$_3^-$, 10 mM NH$_4$NO$_3$, or 5 mM Gln) did not result in a strong repression of *NRT2.1* expression in *chl1-5*, whereas these treatments almost completely abolished it in Col (Figure 6). Reciprocally, transfer of the plants to N-deprived solution failed to derepress this gene in *chl1-5*, whereas the usual transient upregulation was observed in Col (Figure 8). Collectively, these data show that root *NRT2.1* expression in *NRT1.1* mutants is blocked in a derepressed state and, thus, that NRT1.1 is required for correct regulation of *NRT2.1* by N status of the plant. The same conclusion may also be drawn for the activity of the HATS for NO$_3^-$, which appears to be unusually insensitive in *chl1-5* plants to the repression exerted by the presence of NH$_4^+$ in the nutrient solution (Figures 3 and 7). This role of NRT1.1 in controlling both the regulation of the NO$_3^-$ HATS activity and *NRT2.1* expression seems to be quite specific. First, regulation of *AMT1.1* is not affected in *chl1-5* plants (Figure 8). Second, the two other known regulations of *NRT2.1* expression, namely induction by NO$_3^-$
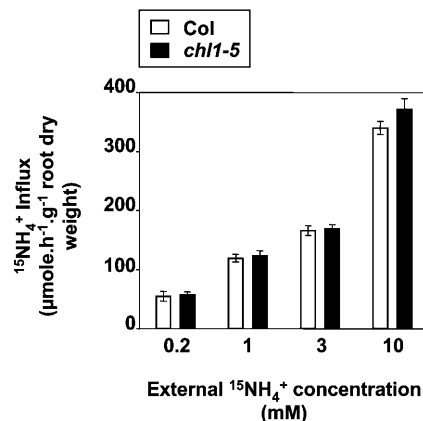
Figure 10. Root ^{15}NH$_4^+$ Influx in Col and *chl1-5* Plants as a Function of the External ^{15}NH$_4^+$ Concentration.

The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH$_4$NO$_3$ as N source. Root ^{15}NH$_4^+$ influx was assayed by 5 min labeling at the external ^{15}NH$_4^+$ concentrations indicated. Results are the mean of 8 to 12 replicates $\pm$ SE.
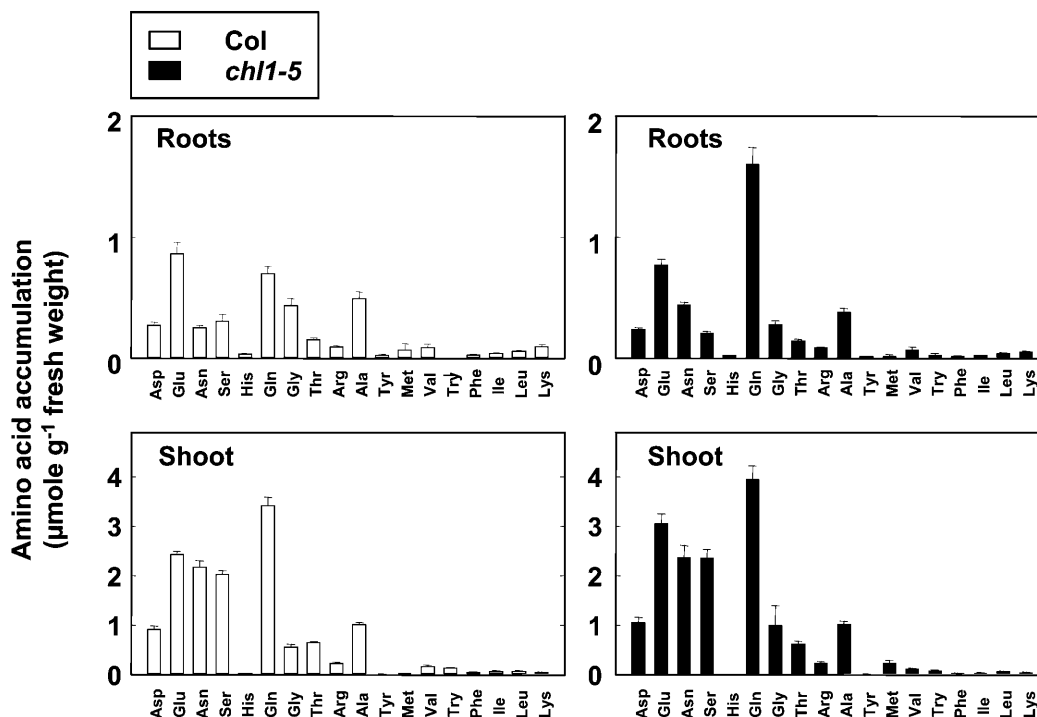
Figure 11. Accumulation of Free Amino Acids in the Roots and Shoot of Col and *chl1-5* Plants.
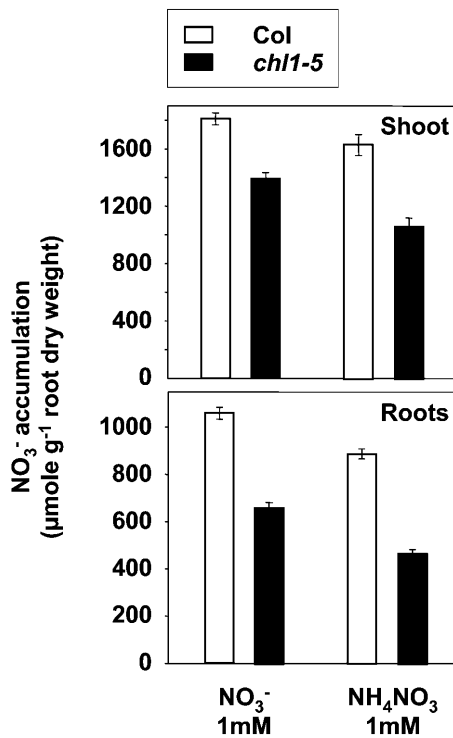
The plants were grown hydroponically for 6 weeks on complete nutrient solution containing 1 mM NH$_4$NO$_3$ as N source. Results are the mean of four replicates ± SE.

(Filleur and Daniel-Vedele, 1999) and regulation by day/night cycle and photosynthates (Lejay et al., 1999, 2003), are also not altered by *NRT1.1* mutation (Figure 9).

The mechanism responsible for the role of NRT1.1 in the regulation of *NRT2.1* expression is unclear. However, the observation that low NO$_3^-$ availability in presence of 1 mM NH$_4^+$ upregulates *NRT2.1* expression in the wild type (Figure 13B) indicates that mutation of *NRT1.1* is not strictly required for preventing downregulation of *NRT2.1* by N metabolites. NRT1.1 is believed to be the main transporter responsible for NO$_3^-$ uptake from mixed N sources (Huang et al., 1996; Touraine and Glass, 1997; Crawford and Glass, 1998). Thus, the two situations that lead to overexpression of *NRT2.1* in the presence of 1 mM NH$_4^+$ (e.g., mutation of *NRT1.1* or decrease in external NO$_3^-$ availability) are both expected to result in a reduced NO$_3^-$ uptake rate. Accordingly, these situations are associated with lowered NO$_3^-$ accumulation in tissues (Figures 12 and 13). This strongly suggests that low NO$_3^-$ uptake rate from mixed NH$_4$NO$_3$ nutrient solution is the actual cause for the upregulation of *NRT2.1*. Because the presence of 1 mM NH$_4^+$ in the medium prevents N deficiency in both wild-type and mutant (see above), this would imply that *NRT2.1* expression is specifically repressed by high NO$_3^-$ uptake rate, independently of the products of NO$_3^-$ assimilation. Hence, one hypothesis would be that two distinct signaling pathways have to be considered for mediating repression of *NRT2.1* by N status of the plant: (1) the well-known feedback repression by N metabolites, related to a specific

reduced N status and mediating the reduced N demand for growth of the plant, and (2) a yet unknown feedback repression by NO$_3^-$ uptake or NO$_3^-$ content of the tissues, related to the NO$_3^-$ status of the plant and mediating a specific NO$_3^-$ demand. The NO$_3^-$ demand signaling would override feedback repression by N metabolites to stimulate *NRT2.1* expression in situations where NO$_3^-$ uptake rate is low in presence of NH$_4^+$ (e.g., in the wild type supplied with nutrient solution at high NH$_4^+$/NO$_3^-$ ratio or in *chl1* mutants).

How might *NRT2.1* be regulated both by NO$_3^-$ induction (Filleur and Daniel-Vedele, 1999; Zhuo et al., 1999) and by repression by high NO$_3^-$ status remains an unanswered question. Opposite direct signaling roles of NO$_3^-$ (induction/repression) in the regulation of its own uptake systems have already been proposed from physiological studies (Siddiqi et al., 1989; King et al., 1993). However, experiments with NR-deficient mutants or using tungstate, a potent NR inhibitor, provided evidence that on NO$_3^-$ as sole N source, *NRT2.1* is predominantly repressed by products of NO$_3^-$ assimilation and not by NO$_3^-$ itself (Krapp et al., 1998; Lejay et al., 1999; Zhuo et al., 1999). On the other hand, it is now well documented that NO$_3^-$ acts both as a positive and a negative signal for the development of the root system, independently of the reduced N status of the plant (Scheible et al., 1997; Stitt, 1999; Zhang et al., 1999). The model proposed for regulation of lateral root development by NO$_3^-$ (Zhang et al., 1999; Zhang and Forde, 2000) is of major interest in our context. It postulates repression of lateral root

Figure 12. Accumulation of NO_3^- in the Roots and Shoot of Col and *chl1-5* Plants.

The plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH_4NO_3 as N source and were either kept on this solution or transferred on another one with 1 mM NO_3^- as N source 1 week before the harvest. Results are the mean of 12 replicates ± SE.

elongation by two separate signaling pathways, one dependent on feedback repression by NO_3^- itself and the other one dependent on feedback repression by reduced N metabolites (e.g., the same dual mechanism as the one we propose above for regulation of *NRT2.1* expression). Furthermore, a local stimulatory effect of NO_3^- has also been documented for lateral root emergence (Zhang and Forde, 2000), showing that NO_3^- can indeed play opposite roles on a specific process, depending on the conditions. Clearly, the hypothesis that *NRT2.1* expression may be upregulated by NO_3^- demand through direct repression by NO_3^- itself deserves particular attention because it creates a strong parallel between the regulatory networks involved in the control of two highly interdependent components of NO_3^- acquisition by the plant (e.g., the NO_3^- uptake systems and the size and architecture of the root system).

It is unclear whether the putative NO_3^- demand signaling triggers upregulation of *NRT2.1* only in presence of NH_4^+ in the external medium or also in other circumstances. For instance, it can also account for the high *NRT2.1* transcript level found in the roots of *chl1-5* plants supplied with NO_3^- as the sole N source (Figure 6) because lowered NO_3^- accumulation in the mutant was also observed in this situation (Figure 12). However, this is in contradiction with the conclusion that *NRT2.1* is predominantly

repressed by downstream N metabolites when plants are supplied with NO_3^- as the sole N source (see above). Furthermore, lack of derepression of *NRT2.1* by N starvation in the *chl1-5* mutant (Figure 8) does not fit well with the hypothesis that mutation of *NRT1.1* alters *NRT2.1* expression through specific regulation by NO_3^- status only. In that case, N starvation should still alleviate feedback repression by reduced N status, mediated by N metabolites, and would result in a further stimulation of *NRT2.1* expression, which is not observed. Alternatively, the lower NO_3^- content in roots of *chl1* mutants (Figures 12 and 13A) may result in a faster loss of *NRT2.1* induction by NO_3^-, which may then prevent any increase in *NRT2.1* transcript level upon transfer of the plants to N-free solution.

Without ruling out the NO_3^- demand signaling as described above, these considerations suggest that other hypotheses could also be envisaged to account for all effects of NRT1.1 mutation on *NRT2.1* expression. In particular, the hypothesis that NRT1.1 plays a direct regulatory role and that its activity generates a signal required for feedback repression of *NRT2.1* by N metabolites also has the potential to explain our results. According to this, mutation of NRT1.1 in the *chl1* mutants would then prevent feedback repression of *NRT2.1* by N metabolites in any situation, thus explaining all *NRT2.1* expression data obtained in these mutants, even when NO_3^- is the sole N source (Figure 6). Furthermore, if not repressed in N replete *chl1* plants, *NRT2.1* obviously cannot be derepressed by N starvation, which then provides a hypothesis for the lack of increase in *NRT2.1* transcript level after transfer of *chl1-5* plants to N-free medium (Figure 8). Finally, direct repression of *NRT2.1* expression by NRT1.1 activity may also account for the fact that *NRT2.1* transcript level is high in wild-type plants supplied with 0.1 mM NO_3^- plus 1 mM NH_4^+, whereas it is low when NO_3^- concentration is increased up to 1 or 10 mM, without modifying that of NH_4^+ (Figure 13B). Indeed, NRT1.1 is dephosphorylated and functions as a low-affinity transporter in plants under high N provision, whereas it is phosphorylated and has a high affinity for NO_3^- in N-limited plants (Liu and Tsay, 2003). Although the phosphorylation status of NRT1.1 has not been investigated under our specific experimental conditions, we can hypothesize that the supply of 1 mM NH_4^+ was sufficient to warrant high N provision to the plants and that, accordingly, NRT1.1 was predominantly in the low-affinity form. If this hypothesis is valid, 0.1 mM NO_3^- in the external medium would have been too low to allow any significant transport activity of NRT1.1, thus preventing generation of the repressive signal for *NRT2.1* expression. By contrast, at 1 or 10 mM external NO_3^-, the low-affinity form of NRT1.1 is significantly or fully active, which then leads to repression of *NRT2.1*. Additional evidence further suggests a signaling role of NRT1.1 in *NRT2.1* regulation. Indeed, one puzzling aspect of our results is that *NRT2.1* expression was dramatically stimulated in the wild type by the increase in external NH_4^+/NO_3^- ratio, with only a small decrease in NO_3^- concentration in roots, and almost no change of this concentration in shoot (Figure 13). Although we cannot exclude a stringent control of *NRT2.1* expression by the NO_3^- demand signaling below a threshold level of NO_3^- accumulation, this may indicate that it is the sensing of the external NO_3^- concentration or of the NO_3^- influx, rather than that of NO_3^- content of the tissues, which is important

A

B
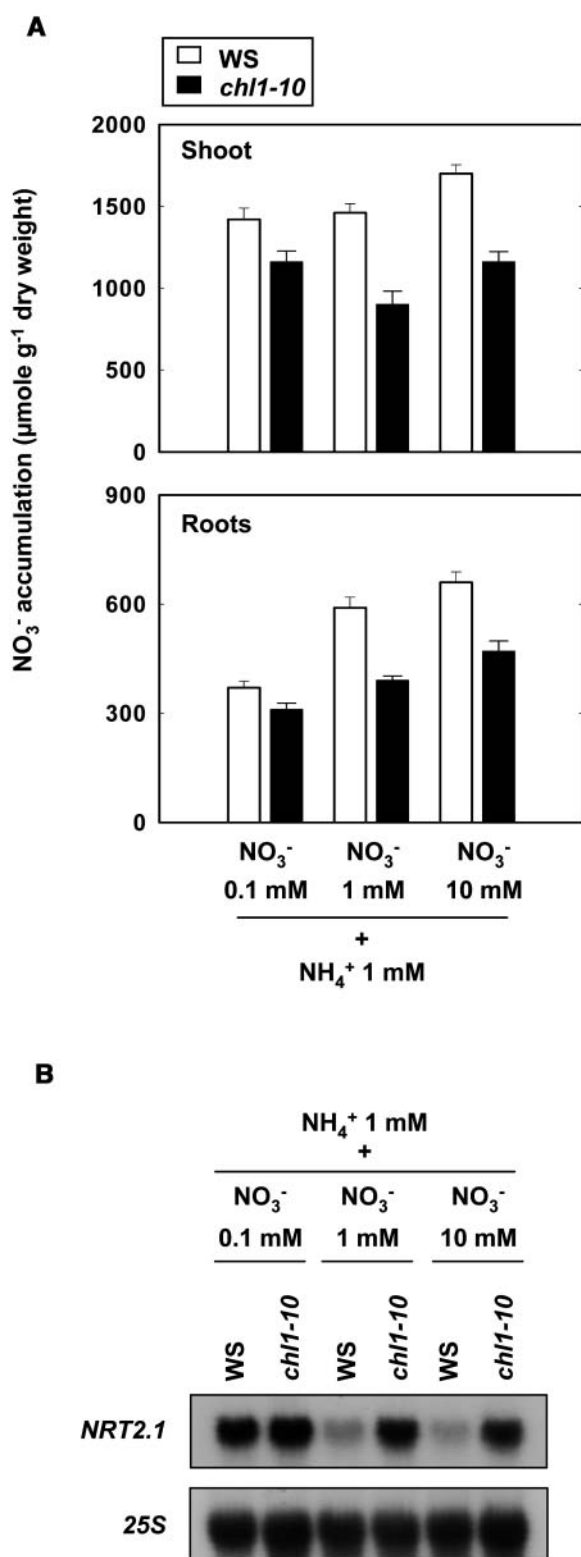
Figure 13. Accumulation of NO_3^- in the Roots and Shoot of Ws and *chl1-10* Plants and Gel Blot Analysis of *NRT2.1* Transcript Accumulation in the Roots of These Plants as a Function of the External NH_4^+/NO_3^- Ratio.

for the regulation of *NRT2.1* in the presence of NH_4^+ in the medium. Because this regulation is strongly altered in *chl1* mutants, NRT1.1 is thus a good candidate for a NO_3^- flux and/ or a NO_3^- concentration sensor. It is now firmly established in both yeast and plants that specific membrane proteins have a dual transport and signaling role (Lalonde et al., 1999). In yeast, various aspects of N signaling are related to the sensing activity of such proteins. For instance, the permease homolog SSY1 is involved in the regulation of the expression of amino acid and peptide transporters by the external N source (Didion et al., 1998; Iraqui et al., 1999), and the high-affinity NH_4^+ transporter MEP2 triggers pseudohyphal growth in conditions of N limitation (Lorenz and Heitman, 1998). Most interestingly, both NRT1.1 and NRT2.1 have been recently proposed to trigger morphological changes in the root system of *A. thaliana*, which are not explained by the purely nutritional role of these proteins (Guo et al., 2001; Rao et al., 2003). Although our data provide additional circumstantial evidence for a sensing function of NRT1.1, much more direct clues are needed for a definite conclusion on this point. Thus, it is not possible yet to exclude any of the two main hypotheses proposed for explaining the surprising role of NRT1.1 in *NRT2.1* regulation. Further analysis of the phenotype of *chl1* mutants is being performed to answer this question.

Whatever mechanism is responsible for the upregulation of *NRT2.1* by low NO_3^- uptake in the presence of ample NH_4^+ supply (NO_3^- demand signaling or lack of NRT1.1-mediated repression), these hypotheses have a strong physiological significance because the ability of the plant to take up NO_3^- in presence of NH_4^+ in the external medium prevents the detrimental effects of pure NH_4^+ nutrition (Salsac et al., 1987; Volk et al., 1992; von Wirén et al., 2000). Indeed, most herbaceous species achieve highest growth rates on a mixed NH_4NO_3 N source, whereas supply of NH_4^+ alone generally results in poor growth and various metabolic disorders (Mehrer and Mohr, 1989; Walch-Liu et al., 2000). Despite its importance, no regulatory mechanism was known to specifically promote NO_3^- uptake from mixed N source. We suggest here that regulation of *NRT2.1* by either NO_3^- demand or NRT1.1-dependent signaling corresponds to such a mechanism. Thus, in addition to being involved in satisfying the plant's N requirements for growth when NO_3^- is the only N source (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999; Cerezo et al., 2001; Gansel et al., 2001), NRT2.1 would also play a key role under mixed nutrition in contributing to maintain a healthy balance between NO_3^- and NH_4^+ uptake.

(A) Accumulation of NO_3^- in the roots and shoot of Ws and *chl1-10* plants.
(B) Gel blot analysis of *NRT2.1* transcript accumulation in the roots of these plants.
The plants were grown hydroponically for 5 weeks on complete nutrient solution containing 1 mM NH_4NO_3 as N source and transferred for 6 d on media containing 1 mM NH_4Cl plus either 0.1, 1, or 10 mM KNO_3. NO_3^- accumulation results are the means of 12 replicates $\pm$ SE.

METHODS

Plant Material and Treatments

All genotypes (Col-0, Ws, Landsberg *erecta, chl1-1, chl1-5, chl1-10*, and *chl1-11*) used in this study were grown hydroponically using the experimental setup described previously (Lejay et al., 1999). Briefly, seeds were sown directly on the surface of wet sand in modified 1.5-mL microcentrifuge tubes, with the bottom replaced by a metal screen. The tubes supporting the seeds were placed on polystyrene floating rafts, on the surface of a 10-liter tank filled with tap water. The culture was then performed in a controlled growth chamber with 8-h/16-h day/night cycle at 24°C/20°C. Light intensity during the light period was at 250 μmol m^{-2} s^{-1}. The basal nutrient solutions supplied to the plants are those described by Gansel et al. (2001) and contained either 1 mM NO$_3^-$ or 1 mM NH$_4$NO$_3$ as N source. For specific experiments involving response to N deprivation or to various N sources, the NH$_4$NO$_3$ solution was used, in which NH$_4$NO$_3$ was either omitted or replaced by other N sources indicated in the figures. One week after sowing, the tap water was replaced by diluted (1/10) basal medium. After one additional week, the plants were supplied with undiluted nutrient solution until the age of 5 weeks when experiments generally began. The nutrient solution was replaced every week during this period. During the experiments, nutrient solutions were renewed daily and adjusted at pH 5.8. Except when the effect of day/night cycle was investigated, all harvests and measurements were done 5 h into the light period.

Characterization of the Genomic Deletion in *chl1-5*

Three consecutive steps of PCR were performed on Col-0 or *chl1-5* genomic DNA to map the deletion, using 15 primer pairs designed from T28K15 and F12F1 BAC sequences. At the end of this process, the right and left borders of the deletion were mapped with 1-kb accuracy each. Then, two oligonucleotides, delF (5′-TATCCTTCACACACATgCATg AC-3′) and delR (5′-AATgCAgTCATgCAgTTTATgCC-3′), with their related genomic sequences separated by 19.4 kb on chromosome 1, were used to amplify the corresponding region in the *chl1-5* mutant. As expected, the large 19.4-kb fragment could not be amplified with Col genomic DNA, but a 1.1-kb fragment was amplified with *chl1-5* genomic DNA using Pfu polymerase (Promega, Madison, WI). The 1.1-kb fragment obtained was cloned using pCR blunt plasmid (Invitrogen, Carlsbad, CA) and DH5α competent cells. DNA was then sequenced by Genome express (Grenoble, France).

Isolation of *chl1-10* and *chl1-11* Chlorate-Resistant Mutants

The *chl1-10* and *chl1-11* chlorate resistant mutants were isolated from the INRA collection of T-DNA insertion lines of *Arabidopsis thaliana* (ecotype Ws, Versailles, France). The screen was done on 6-d-old seedlings germinated on soil. Chlorate treatment was performed by subirrigating plants every 2 d during 15 d with a nutrient solution containing 2 mM KClO$_3$ and 2 mM NH$_4$NO$_3$ as sole nitrogen source. DNA gel blots performed using probes for right and left borders of the T-DNA suggested that *chl1-10* and *chl1-11* carry one and four insertions, respectively. An allelism test performed with the *chl1-5* mutant indicated that two mutants, named *chl1-10* and *chl1-11*, belong to the *chl1* complementation group. DNA gel blots performed using a specific probe for *NRT1.1* showed that the structure of this gene was disturbed in both *chl1-10* and *chl1-11* mutants. The disruption of the *NRT1.1* gene in the *chl1-10* mutant was characterized by PCR amplification and sequencing of the T-DNA flanking sequences using primers specific to both left and right T-DNA borders (5′-GTCGGCTATTGGTAATAGGA-3′ and 5′-CCACAGGCC-GTCGAGTTTT-3′, respectively) and *NRT1.1* flanking genomic sequence (5′-GACGTAGAAGACTGCCATCGATG-3′ and 5′-TTTGTCATGCATGT-GTGTGAAGG-3′, respectively).

SAGE Protocol

The root samples harvested from Col-0 and *chl1-5* NH$_4$NO$_3$-grown plants were stored at −80°C before total RNA extraction. The SAGE libraries were obtained from 100 μg of total RNA, using SAGE protocol described by Virlon et al. (1999), with the difference that the anchoring enzyme was *Mbo*I (New England Biolabs, Beverly, MA) instead of *Sau*3AI. Poly(A) RNAs were isolated from 100 μg of total RNA using Dynabeads mRNA direct kit (Dynal, Brown Deer, WI) based on oligo(dT)$_{25}$ bound covalently to magnetic beads. cDNA were synthesized directly on the beads, and all enzymatic steps needed before digestion by *Bsm*FI were performed on cDNA linked to the beads. All oligonucleotides, with sequences and modifications identical to Virlon et al. (1999), were from Eurobio (Les Ulis, France).

Final concatemers were cloned in pBluescript II KS− from Stratagene (La Jolla, CA), digested by *Eco*RV, dephosphorylated, and purified on agarose gel. Ligation was performed overnight at 16°C and ElectroMAX DH10B *Escherichia coli* cells (Life Technologies, Cleveland, OH) were then used for transformation by electroporation. Sequencing was performed as described previously (Fizames et al., 2004) in the Department Genome et Développement des Plantes (University of Perpignan, France) and Genome Express (Grenoble, France). Altogether, 1176 runs of sequencing were needed to obtain the 28,952 tags of the *chl1-5* root SAGE library and 1335 runs for the 31,354 tags of the Col-0 root SAGE library.

SAGE Data Analysis

The whole procedure developed to obtain transcript profiles from concatemer sequences is described in Fizames et al. (2004). Briefly, experimental tag sequences were extracted from the concatemer sequences using DIGITAG software (Piquemal et al., 2002). Tag to gene assignment was then performed by matching the sequences of the experimental tags with those of virtual tags extracted from 26,620 annotated genes of the *A. thaliana* genome (ftp://ftpmips.gsf.de/cress/arabidna/arabi_genomic-plus500_v111102.gz), taking into account the coding sequence plus 400 bp 5′ and 3′ extensions. We have previously determined (Fizames et al., 2004) that this procedure allows the identification of the transcripts corresponding to ∼60% of the tags found experimentally, with a specificity of 85% (only 15% of the experimental tags match more than one gene), and a reliability of 88% (only 12% of the experimental tags are assigned to wrong genes). The statistical analysis of SAGE data for identification of genes differentially expressed between roots of Col-0 and *chl1-5* plants was performed as described in Piquemal et al. (2002).

RNA Extraction and RNA Gel Blot Analysis

Total RNA extraction was performed on roots as described previously (Lobreaux et al., 1992). For RNA gel blot analysis, total RNA (15 μg) was separated by electrophoresis on 3-(*N*-morpholino)-propanesulfonic acid formaldehyde agarose gel and blotted on nylon membrane (Hybond N$^+$; Amersham Pharmacia Biotech, Buckinghamshire, UK). Membranes were prehybridized for 2 h at 65°C in Church buffer (0.5 M NaHPO$_4$, 1% BSA, and 7% SDS, pH 7.2, with H$_3$PO$_4$). Hybridizations were performed overnight at 65°C after addition of a randomly primed ^{32}P-labeled cDNA probe in the prehybridization buffer. Membranes were washed twice at room temperature for 2 min and twice at 65°C for 15 min with 0.5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS. DNA probes used in this study correspond to full-length cDNAs. A 25S rRNA probe was used as reference for quantification achieved using a PhosphorImager (Storm; Molecular Dynamics, Sunnyvale, CA).

^{15}NO$_3^-$ and ^{15}NH$_4^+$ Uptake

Root ^{15}NO$_3^-$ or ^{15}NH$_4^+$ influxes were assayed as described by Delhon et al. (1995) for NO$_3^-$ and by Gazzarrini et al. (1999) for NH$_4^+$. Briefly, the

plants were sequentially transferred to 0.1 mM $CaSO_4$ for 1 min, to complete nutrient solution, pH 5.8, containing either $^{15}NO_3^-$ or $^{15}NH_4^+$ (99% atom excess ^{15}N) for 5 min at the concentrations indicated in the figures, and finally to 0.1 mM $CaSO_4$ for 1 min. Roots were then separated from shoots, and the organs dried at 70°C for 48 h. After determination of their dry weight, the samples were analyzed for total N and atom percentage ^{15}N using a continuous-flow isotope ratio mass spectrometer coupled with a C/N elemental analyzer (model ANCA-MS; PDZ Europa, Crewe, UK) as described in Clarkson et al. (1996). Each influx value is the mean of 6 to 12 replicates.

Amino Acid and NO_3^- Analysis

After harvest of the plants, roots and shoot were separated and stored either at −20°C for amino acid analysis or dried for 48 h at 70°C for NO_3^- analysis. Free amino acids were extracted from 0.5 g of frozen tissue by grinding in 2 mL of EtOH. The extracts were then left for 1 h at 4°C before centrifugation for 10 min at 2400 rpm and at 4°C. Supernatants were recovered and pellets were subjected to three additional extraction steps identical to the first one, except that these were performed in 1 mL of 80% EtOH, 60% EtOH, and water, respectively. The four supernatants from the same sample were pooled, and an aliquot of this solution filtered (0.45 μm) for amino acid quantification by HPLC (gradient pump SP8800 [Spectra Physics, Mountain View, CA], fluorimeter 821-FP [Jasco, Easton, MD], and SP4270 integrator piloted by SP-LABNET software [Spectra Physics]).

Extraction of NO_3^- was performed in 0.1 N HCl overnight at 4°C. The NO_3^- concentration in the extracts was determined colorimetrically at 540 nm after reduction to NO_2^- on a Cd column and addition of sulfanylamide and N-naphtyl-ethylene-diamine-dichloride.

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